AVIAN DISEASES 52:136–138, 2008

Research Note—

The Effects of Increasing Sodium Chloride Concentration on *Mycoplasma gallisepticum* Vaccine Survival in Solution

S. A. Leigh,[A] J. D. Evans, S. L. Branton, and S. D. Collier

USDA, ARS South Central Poultry Research Unit, Mississippi State, MS 39762

Received 5 April 2007; Accepted and published ahead of print 23 August 2007

SUMMARY. Lyophilized *Mycoplasma gallisepticum* (MG) vaccines are generally rehydrated and diluted with distilled or chlorine-free water as per manufacturer recommendations. However, as mycoplasma species lack a cell wall, this can lead to decreased viability of live vaccine during administration. The ability of phosphate-buffered saline (PBS) to prevent losses in live vaccine viability was examined. It was shown that a concentration of 1× PBS prevented the two–fourfold decrease in MG viability seen when the vaccines were diluted with water alone.

RESUMEN. *Nota de Investigación*—Efectos del aumento de la concentración de cloruro de sodio sobre la supervivencia en solución de la vacuna de *Mycoplasma gallisepticum*.

Las vacunas liofilizadas de *Mycoplasma gallisepticum* generalmente son rehidratadas y diluidas con agua destilada o con agua libre de cloro de acuerdo con las instrucciones del fabricante. Sin embargo, debido a que las especies de micoplasma carecen de pared celular, esto puede resultar en una disminución de la viabilidad de las vacunas vivas durante su administración. Se estudió la capacidad de la solución salina fosfatada y buferada para prevenir las pérdidas de viabilidad de la vacuna viva. Se encontró que una concentración de un volumen de solución salina buferada y fosfatada previno la disminución de la viabilidad de la vacuna de *Mycoplasma gallisepticum* de dos a cuatro veces comparado con el uso de agua destilada sóla.

Key words: *Mycoplasma gallisepticum*, phosphate-buffered saline, FVAX-MG®, Mycovac-L®

Abbreviations: CCU_{50} = color change units calculated to a 50% endpoint; MG = *Mycoplasma gallisepticum*; PBS = phosphate-buffered saline

Mycoplasma gallisepticum (MG) causes chronic respiratory disease of chickens and infectious sinusitis of turkeys (6). Three live MG vaccines are currently available. Two of the vaccines are supplied as lyophilized pellets and are often rehydrated and diluted with nonchlorinated water or distilled water prior to application (1,2). Although these conditions may be sufficient for most live vaccines, their effects on live mycoplasma vaccine viability are in question.

The main reason that the usage of distilled or nonchlorinated water to rehydrate and dilute MG vaccines is a concern is that, unlike most bacteria, mycoplasmas lack a cell wall (10). This leaves them susceptible to osmotic lysis when the vaccine is diluted in distilled water (13). Although *M. gallisepticum* is known to be resistant to osmotic lysis compared to other mycoplasma species (7,13), this does not mean that it is as resistant as bacteria with a cell wall. Previous work has demonstrated that the F strain of MG was stable in water for at least 2 hr (5). However, this work was done before commercial live vaccines were available, and the results were only accurate to one log difference at best. In order to address the effects of salt on MG vaccine survival, phosphate-buffered saline (PBS) at various concentrations was studied to see if it could stabilize MG vaccine viability compared to distilled water.

MATERIALS AND METHODS

Vaccine strains. *Mycoplasma gallisepticum* (MG) vaccines FVAX-MG® (Schering Plough Animal Health, Omaha, NE) and Mycovac-L® (Intervet Inc., Millsboro, DE) were obtained from their respective commercial sources. The vaccines were stored at 4 C. The same respective vaccine lot for each vaccine was used for all experiments.

Conditions for testing salt concentrations. All solutions were made with distilled, deionized water. The same bottle of powdered PBS concentrate (Thermo Fisher Scientific, Waltham, MA) was used for all experiments. Water-only control (0× PBS) solution was buffered with 0.5 mM sodium phosphate, and the pH was adjusted to 7.3 to match the approximate pH of the PBS containing solutions. For each experiment, the lyophilized vaccine pellet was rehydrated to a volume of 10 ml with the 0× PBS solution. It was then immediately diluted into the individual PBS containing test solutions at a rate of 1 dose per 800 µl. This dilution ratio was used to coincide with dilution ratios used by some poultry producers. Experiments were performed at room temperature with the use of either two or three independent replicates of each condition. Samples were removed at 15-, 30-, and 60-min time points.

CCU_{50} and statistics. MG survival was measured by determining the number of color change units (CCU_{50}) per ml. At each time point, 100 µl was removed from each sample and immediately diluted in Frey's medium (4). CCU_{50} was determined as described previously (9). Briefly, serial 10-fold dilutions were incubated at 37 C in a sealed microtiter plate. CCU_{50} for each sample was calculated by the method of Reed and Muench (11). The results are expressed as percent survival, determined by dividing the CCU_{50} obtained for each sample/time point by the average starting ($t = 0$) CCU_{50} of the diluted vaccine. Significance of differences was determined with the use of an unpaired, two-tailed *t*-test. Differences were considered to be significant if $P \leq 0.05$.

RESULTS

The use of PBS for dilution of lyophilized vaccines was investigated for its ability to increase vaccine survival in solution.

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

[A]Corresponding author. E-mail: sleigh@msa-msstate.ars.usda.gov

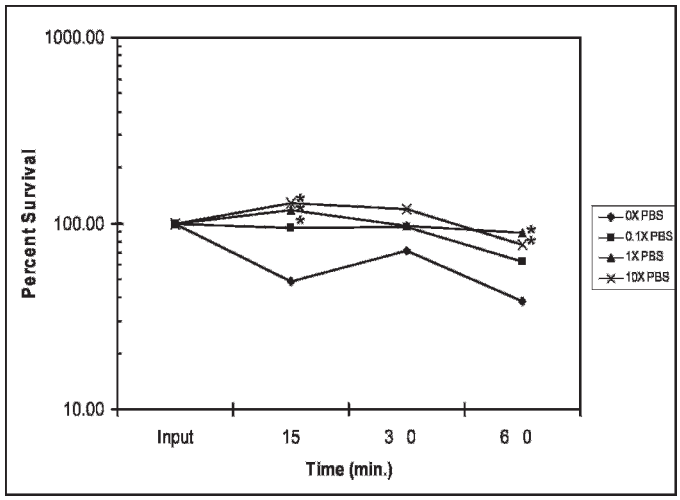

Fig. 1. Survival of FVAX-MG® in: 0× PBS (◆), 0.1× PBS (■), 1× PBS (▲), and 10× PBS (×). Significant differences in data points ($P \leq 0.05$) compared to 0× PBS control are labeled (*).

Results for FVAX-MG® show that addition of the PBS concentrate at all concentrations increases vaccine survival over the 0× PBS control (Fig. 1). No statistical difference was seen for any of the samples at 30 min, due to a repeatable spike in survival of the 0× PBS control. At 15 min, all three PBS-containing solutions were significantly different from the 0× PBS control. By 60 min, the survival of the vaccine in all of the solutions had decreased, but survival of vaccine diluted in the 1× and 10× PBS solutions was significantly better than that of the 0× PBS control.

Results for Mycovac-L® were similar to those for FVAX-MG® (Fig. 2). There was no significant difference between the 0.1× and 0× PBS samples at any time point. The 1× PBS sample gave the best results, and was significantly different from the 0× PBS sample at all time points. Although there was no statistically significant difference between the 1× and 10× concentrations, the 10× concentration was not significantly different from the 0× PBS control at 30 min, unlike the 1× concentration.

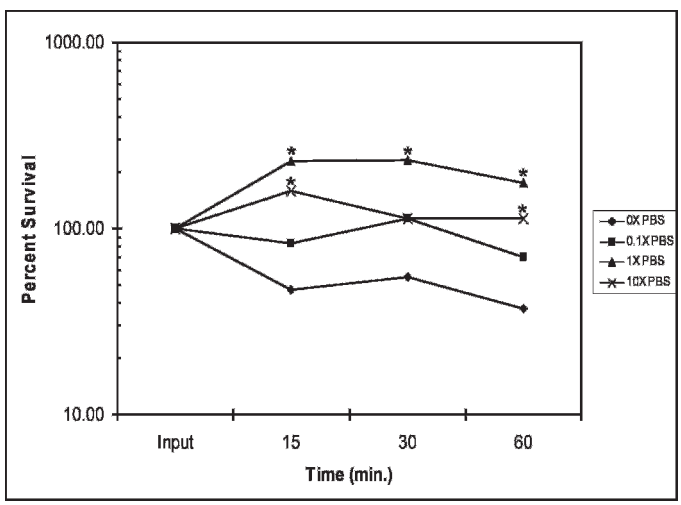

Fig. 2. Results for Mycovac-L® in: 0× PBS (◆), 0.1× PBS (■), 1× PBS (▲), and 10× PBS (×). Significant differences in data points ($P \leq 0.05$) compared to 0× PBS control are labeled (*).

DISCUSSION

These results suggest that dilution of rehydrated MG vaccines in 1× PBS provided the most cost-effective and reliable results. The 10× PBS sample appeared to give nearly equivalent results when compared to 1× PBS. However, it also increases the cost of the PBS concentrate 10-fold, and would result in the unnecessary deposition of large quantities of salt onto cages and equipment.

In some situations, 0.1× PBS may provide equivalent benefits for FVAX-MG® when compared to 1× PBS, in particular when the vaccine was used within 30 min of reconstitution. The results for FVAX-MG® at 0.1× PBS may be skewed compared to the results for Mycovac-L®. For the vaccine lots used in this experiment, FVAX-MG® contained on average 2.07 g per vial with only 1000 doses. The Mycovac-L® vial contained 1.33 g per 2000 doses. The extra material in the FVAX-MG® lyophilized vaccine pellet may contribute sufficient material to increase its survival at the low salt concentration (0.1× PBS) as compared to Mycovac-L®. Alternatively, it is possible that differences in lyophilization procedure, content of pellet material, and/or the genetics of the two species may be the cause of the differences seen. One unexpected result was that both vaccines have much greater survival with 1× and 10× PBS than the 100% percent survival value set using the $t = 0$ starting value. It is possible that this is an artifact found during rehydration of lyophilized vaccines or due to clumping of the rehydrated bacteria, resulting in an artificially low count of the number of bacteria at the beginning of the experiment.

There are differences between this work and the results presented by Kleven (5) and Christensen *et al.* (3). Kleven reported that the F strain of MG showed no decrease in viability over 24 hr at room temperature in PBS (5). The differences reported may be due to less accuracy in measured MG survival in the paper by Kleven. The differences noted in this work would have been too small to detect by the methods Kleven used. In contrast to the work by Kleven, Christensen *et al.* (3) reported a rapid decline in MG strain PG31 survival after just 3 hr in PBS at room temperature with complete loss of viability in 6 hr. However, there are no data points between the initial MG concentration and the 3-hr time point (3). If the decrease in MG viability came after the first 60 min, it would not have been seen in the results reported here.

There is some concern using PBS or other salt-containing solutions for dilution of live mycoplasma vaccines. Mycoplasma species use an active sodium efflux system to maintain proper volume regulation (8,12). As ATP levels in the cell decrease, the ability to export sodium ions actively ceases and the cells swell and lyse (8). Because of the relatively short time interval, 10–48 min (2) that mycoplasma are suspended in the PBS solution before and during spraying of a 75,000 bird houses, this does not appear to be a major problem. It could, however, account for the drop in cell viability between 30 and 60 min. This sensitivity to sodium-containing solutions may have caused the differences in results reported by Kleven and Christensen *et al.* Kleven diluted mycoplasma culture in PBS at a rate of 100 ml culture medium per 2 gal of solution (5), whereas Christensen *et al.* washed the mycoplasma in sterile distilled deionized water prior to use (3). The addition of 1–10 mM glucose has been reported to restore sodium efflux and proper cell volume (8,12). It is probable that the presence of culture medium in Kleven's work had a similar effect on maintaining MG viability, although differences in the MG strains may also account for the different results. It is unknown what materials are in lyophilized MG vaccine pellets, but the difference in pellet composition may also contribute to the difference seen in Mycovac-L® versus FVAX-MG® survival.

REFERENCES

1. Bermudez, A. J., and B. Stewart-Brown. Disease prevention and diagnosis. In: Diseases of poultry, 11th ed. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne, eds. Iowa State Press, Ames, IA. pp. 17–55. 2003.

2. Branton, S. L., W. B. Roush, B. D. Lott, J. D. Evans, W. A. Dozier III, S. D. Collier, S. M. D. Bearson, B. L. Bearson, and G. T. Pharr. A self-propelled, constant-speed spray vaccinator for commercial layer chickens. Avian Dis. 49:147–151. 2005.

3. Christiansen, K. H., C. A. Yarari, A. J. McBain, and J. M. Bradbury. Investigations into the survival of *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Mycoplasma iowae* on materials found in the poultry house environment. Avian Pathology 23:127–143. 1994.

4. Frey, M. C., R. P. Hanson, and D. P. Anderson. A medium for the isolation of avian mycoplasma. Am. J. Vet. Res. 29:2164–2171. 1968.

5. Kleven, S. H. Stability of the F strain of *Mycoplasma gallisepticum* in various diluents at 4, 22, and 37 C. Avian Dis. 29:1266–1268. 1985.

6. Ley, D. H. *Mycoplasma gallisepticum* infection. In: Diseases of poultry, 11th ed. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne, eds. Iowa State Press, Ames, IA. pp. 722–744. 2003.

7. Lim, P., B. B. Sears, and K. L. Klomparens. Membrane properties of a plant-pathogen mycoplasmalike organism. J. Bacteriol. 174:682–686. 1992.

8. Linker, C., and T. H. Wilson. Cell volume regulation in *Mycoplasma gallisepticum*. J. Bacteriol. 163:1243–1249. 1985.

9. Masover, G. K., R. P. Mischak, and L. Hayflick. Some effects of growth medium composition on the antigenicity of a T-strain mycoplasma. Infect. Immun. 11:530–539. 1975.

10. Razin, S. Mycoplasma taxonomy and ecology. In: Mycoplasma molecular biology and pathogenicity. J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds. American Society for Microbiology, Washington, D.C. pp. 3–22. 1992.

11. Reed, L. J., and H. Muench. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497. 1938.

12. Shirvan, M. H., S. Schuldiner, and S. Rottem. Role of Na^+ cycle in cell volume regulation of *Mycoplasma gallisepticum*. J. Bacteriol. 171:4410–4416. 1989.

13. Wieslander, Å., M. J. Boyer, and H. Wróblewski. Membrane protein structure. In: Mycoplasmas molecular biology and pathogenesis. J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman, eds. American Society for Microbiology, Washington, D.C. pp. 93–112. 1992.